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TITLE: ANTI-ED-B ANTIBODY

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1. TITLE OF INVENTION

Anti-ED-B antibody

2. PATENT CLAIM

[Claim 1]

Anti-ED-B antibody which characteristically possesses the antigen determining group within the amino acid sequences shown by the formulae (1), (2), and (3) below.

(1) : EGIPIFEDFVDSSVGY

(2) : YTVTGLEPGIDYDIS

(3) : NGGESAPTTLTQQT

3. DETAILED EXPLANATION OF INVENTION

[Industrial Utilization Field]

The present invention relates to the novel antigen against the fibronectin (FN), particularly to the FN type comprised in cancer organs.

[Conventional Techniques]

The FN is the multi-functional protein which is widely distributed in various organs and body fluid and is known to contribute to the various biological phenomena such as cell transportation, differentiation, multiplication, and canceration as the cell adhesive factor (Kiyotoshi Sekiguchi, Cell Engineering, Vol. 4, No. 6, p485 - 497 (1985)).

Further, the said FN has been known to exist as two molecular types: those that exist in the matrix outside of cell (cell type FN: cFN) and FN within blood plasma (plasma type FN: pFN). Such the variety in these FN molecules has been clarified to be caused by the alternative splicing of the gene initial transcript product. There are three regions which receive such the alternative splicing: ED-A, ED-B, and IIIcs. By the

combination of the expression in these regions, many molecular species are to be generated.

On the other hand, the FN comprised in the cancer organs (below abbreviated as "cancerous FN") exhibits the unordinary high expression of the said ED-B and is known as the FN having ED-B comprising 91 amino acids (Luciano Zardi, et al., The EMBO Journal, Vol. 6, No. 6, p. 2337 - 2342 (1987)).

[Problems to be solved by the Invention]

Under such the condition, this field anticipates the measures to proceed the research of the said cancerous FN at the molecular level and to enable the measurement (detection) and purification specific to this molecular species, and thus to enable the cancer diagnosis.

The purpose of the present invention is to offer the measure to meet the said expectation. In other words, the present invention aims: to offer the antibody which specifically recognizes the said ED-B, thus possesses the specific reactivity with the cancerous FN; to offer the peptide related to the said ED-B, particularly a hapten for the manufacturing of the said antibody and the specific peptide which can be a tracer for the measurement of the cancerous FN; and further to offer the technique to measure the desired cancerous FN or ED-B by utilizing these.

[Methods to Solve the Problems]

According to the present invention, the anti-ED-B antibody which characteristically possesses the antigen determining group within the amino acid sequences shown by the formulae (1), (2), and (3) below, is offered.

(1) : E G I P I F E D F V D S S V G Y

(2) : Y T V T G L E P G I D Y D I S

(3) : N G G E S A P T T L T Q Q T

Further, the present invention offers the ED-B peptide which is expressed by the amino acid sequences shown by the said formulae (1), (2), and (3).

In the present specification above and below, when amino acids, peptides, protective groups, active groups, and others are expressed by the abbreviated codes, the regulation by IUPAC and the commodity codes in this field are used. These examples are listed below. Further, when the optical isomers may exist for amino acids, it usually indicates L form unless specifically expressed.

A: alanine	D: asparaginic acid	N: asparagine	I: isoleucine
L: leucine	G: glycine	P: proline	S: serine
E: glutamic acid	Q: glutamine		T: threonine
F: phenyl alanine	V: valine	C: cysteine	Y: tyrosine

Tos: p- toluene sulfonyl group
Boc: tertiary butoxy carbonyl group
Bzl: benzyl group
MBzl: p- methoxy benzyl group

OBzl: benzyl oxy group Cl_2 Bzl: 2, 6-dichloro benzyl group
Cl-Z: 2- chlorobenzyl oxy carbonyl group
Br-2: 2- bromobenzyl oxy carbonyl group.

The said specific anti-ED-B antibody offered by the present invention is the antibody which recognizes the antigen section specific to ED-B, and characteristically possesses the specific reaction to the ED-B or the FN having the said region, i.e., to the cancerous FN, and is characterized by not presenting the cross reactivity towards the said other molecular type FNs such as cFN and pFN.

Therefore, the antibody of the present invention can be utilized as the specific antibody at the immunity measurement of ED-B or cancerous FN and the easy measurement method with high sensitivity and high precision can be established by this. Further, the establishment of the said measurement method can offer the cancer screening and diagnostic technique and is also extremely useful for the basic research on the carcinogenic mechanism research and analysis.

Further, the said antibody of the present invention is also useful; for the immunological purification of the said ED-B and cancerous FN by, for example, affinity chromatography.

Further, the said specific peptide (ED-B peptide) offered by the present invention is constructed with the antigen section specific to ED-B, therefore, this is useful as the hapten for the manufacturing of the said anti-ED-B antibody and also can be utilized as the tracer (labeled body) at the said measurement method.

Below, the manufacturing method of the antibody of the present invention is interpreted in details.

The antibody of the present invention can be manufactured by the normal method by using the peptide or protein at least having the amino acid sequences expressed by the said formulae (1), (2), and (3) as the immunogen. For example, there are: the method to produce the desired antibody within the living body by administering (immunizing) a mammal with the said antigen; and the method to form the hybridmer of the plasma cell (immunocyte) of the mammal immunized by the said immunogen and the plasma cytoma cell of the mammal, and then to select and cultivate the clone which produces the desired antibody (monoclonal antibody).

At the manufacturing of the antibody of the present invention, the said peptide or protein used as the immunogen is not limited to any specifics as long as they at least comprise the amino acid sequences expressed by the said formulae (1), (2), and (3). For example, the cancerous FN prepared from the cancer organs, the ED-B region or its fragment from these cancerous FN, and synthetic peptide having the said specific amino acid sequence may be employed. Among these, that obtained by using the ED-B of the present invention as the hapten is most desirable.

In the said method, the mammals which are immunized are not limited to any specific types. However, when the antibody of the present invention is manufactured by using the hybridmer, they should be desirably selected by considering the compatibility with the plasma cytoma cell used for cell fusion, and generally mouse and rat are advantageously employed.

In the said antibody manufacturing method, immunization can be executed by the general method, for example, by administering the immunogen to mammals through the intravenous, endermic, hypodermic, or abdominal injection. In more concrete, the immunogen is combined with the ordinal adjuvant as desired and it is administered to the test animal several times every 2 to 14 days. The said administration amount is, for example, about 10 to 100 μg for mouse and 0.2 to 2.0 mg for domestic rabbit. For the sampling of the antibody, blood sample is collected from the immunized animal after one to two weeks since the said last administration and serum is separated after the centrifugal separation. Further as the immunocyte used for the manufacturing of the said monoclonal antibody, the use of the spleen cell collected after about three days since the said last administration is desirable.

The plasma cytoma cell of the mammal used as the other parent cell fused with the said immunocyte may be the various types which are already known such as myeloma cells: p3 (p3 / x63~ Ag 8, Nature, 256, 495 - 497 (1975)); p3-U1 (Current Topics in Microbiology and Immunology, 81, 1 - 7 (1978)); NS-1 (Eur. J. Immunol., 6, 511 - 519 (1976)); MPC-11 (Cell, 8, 405 - 415 (1976)); SP2/O (Nature, 276, 269 - 270 (1978)); FO (J. Immunol. Meth., 35, 1 - 21 (1980)); x 63. 6. 5. 3 (J. Immunol., 123, 1548 - 1550 (1979)); S194 (J. Exp. Med., 148, 313 - 323 (1978)); and R 210 of Rat (Nature, 277, 131 - 133 (1979)).

The fusion reaction of the said immunocyte and the plasma cytoma cell can be carried out by following the known methods such as the method by Milstein (Method in Enzymology, vol. 73, pp 3 (1981)). In more concrete, the said fusion reaction can be executed in the ordinary medium under the existence of the ordinary fusion promoters such as polyethylene glycol (PEG) and Sendai virus (HVJ). The medium may be further added with the co-agent such as dimethyl sulfoxide in order to increase the fusion efficiency. The usage ratio of the immunocyte and the plasma cytoma cell is same as in the ordinary method. For example, about 1 to 10 times of immunocytes are employed against the plasma cytoma cell. As the medium for the fusion reaction, various types which are ordinarily used for the multiplication of the said plasma cytoma cell, such as RPMI- 1640 medium, MEM medium, and others generally used as the medium for this type cell, are exemplified. Such the media should be used by eliminating the serum co-solution such as bovine fetus serum (FCS). The fusion is carried out by mixing the certain amounts of the said immunocytes and plasma cytoma cells well within the said culture and by adding the PEG solution preliminarily heated to about 37 °C and having the average molecular weight of, for example, 1000 to 6000, to the culture ordinarily at the concentration of about 30 to 60 w/v%. Then, the desired hybridmer is formed by repeating the following procedures: adding the appropriate media consecutively, centrifuging, and eliminating the supernatant solution.

The separation of the obtained desired hybridmer is carried out by cultivating it in the ordinary medium used for selection, such as the HAT medium (the medium comprising hypoxanthine, aminopterin, and thymidine). The cultivation in the said HAT medium should be carried out for the time period sufficient enough for the cells besides the desired hybridmer (such as non-fused cells) to die out, ordinarily for several days to several weeks. The hybridmer obtained as above is provided for the search of the aimed antibody and for the single cloning by the ordinal limiting dilution method.

The search of the aimed antibody producing strain is carried out by following the various methods generally used for the detection of antibody such as the ELISA method (Engvall. E., Meth. Enzymol., 70, 419 - 439 (1980)), plaque method, spot method, agglutination method, Ouchterlony method, and the radio immunoassay (RIA) method ("Hybridmer Method and Monoclonal antibody" published by R&D Planning Co., Ltd., pp 30 - 53, March 5, 1982). For this search, the use of the said ED-B peptide of the present invention is suitable.

The hybridmer which was obtained as above and produces the desired monoclonal antibody of the present invention can be sub-cultivated in the ordinary medium and may be stored for a long time within liquid nitrogen.

The collection of the antibody of the present invention from the said hybridmer employs: the method to obtain as the culture supernatant after cultivating the said hybridmer according to the normal method; and the method to obtain as ascites by administering the hybridmer to the mammal having the compatibility after the multiplication. The former is suitable to obtain the high purity antibody and the latter is suitable for the mass production of the antibody.

Further, the antibody obtained as above can be further purified by the ordinary measures such as salting out, gel filtration, and affinity chromatography.

The anti-ED-B antibody of the present invention can be manufactured as above.

Below, the ED-B peptide of the present invention and the utilization are described in details.

The ED-B peptide of the present invention can be manufactured easily by the simple procedure according to the ordinary chemical synthesis methods by utilizing the easily available commercial amino acids. The said chemical synthesis methods are carried out by the ordinary peptide synthesis methods, more in concrete by following the methods described in "The Peptides", Vol. 1 (1966) (Schr★der and Luhke, Academic Press, New York, USA) or "Peptide Synthesis" (Izumiya, et., al., Maruzen (1975)). Examples of these methods are: the azide method, chloride method, acid anhydride method, mixed acid anhydride method, DCC method, active ester method (p-nitro phenyl ester method, N- hydroxy succinic acid imide ester method, and cyano methyl ester method), the method to use Woodward's reagent K, carbodiimidazole

method, redox method, and DCC/ ateitive **[Note from the Translator]** (HONB, HOBT, HOSu) method. In the said methods, either the solid phase synthesis or liquid phase synthesis may be applicable. The peptide of the present invention is usually manufactured by following the said general peptide synthesis methods: the stepwise method which condenses the terminal amino acid with one amino acid at time; and the coupling method which uses several fragments.

Further in details, when the solid phase synthesis method is employed, C terminal amino acid (protected amino acid) is bound to the insoluble carrier through its carboxyl group. The insoluble carrier is not limited to any specific types as long as it has the bonding property with the reactive carboxyl group. For example, halogeno-methyl resins such as chloromethyl resin and bromomethyl resin, hydroxy methyl resin, phenol resin, and the resin converted to tert- alkyl oxy carbonyl hydrazide may be utilized. Then after removing the amino protective group, amino group protected amino acids are bound in the order according to the amino acid sequence of the aimed peptide through the condensation reaction of the reactive amino group and the reactive carboxyl group, one step at a time. After forming the total sequence, the peptide is removed from the insoluble carrier to obtain the peptide of the present invention.

In the said various methods, it is desirable for each amino acid having side chain functional groups such as Y, E, T, C, and S to have its side chain functional group protected. They can be protected by the ordinary protective groups and the protective groups are removed after the completion of the reaction. Further, the functional groups concerning to the reaction are usually activated. These reaction methods are well known and the reagents used for them are appropriately selected from the well known types.

Examples of the amino acid protective groups are: benzyl oxy carbonyl, Boc, tert-amyl oxy carbonyl, iso-bornyl oxy carbonyl, p- methoxy benzyl oxy carbonyl, Cl-2, adamantyl oxy carbonyl, trifluoro acetyl, phthalyl, formyl, o- nitrophenyl sulfenyl **[Note from the Translator-2]**, and diphenyl phosphino thioyl groups.

Examples of the carboxyl group protective groups are: the groups which can form: alkyl ester (alkyl esters of methyl, ethyl, propyl, butyl, and tert- butyl), Bzl ester, p-nitrobenzyl ester, HBzl ester, p- chlorobenzyl ester, benzhydryl ester, carbobenzoxy-hydrazide, tert- butyl oxy carbonyl hydrazide, and trityl hydrazide.

The hydroxyl groups of S and T may be protected by turning them to ester or ether, however, they may not be necessarily protected. The group suitable for this conversion to ester are: lower alkanoyl group such as acetyl, aloyl group such as benzoyl, and the groups induced from carbonic acid such as benzoyl oxy carbonyl and ethyl oxy carbonyl. Further the group suitable for the conversion to ether are: benzyl, tetrahydro pyranyl, and tert- butyl groups.

Examples of the protective group for hydroxyl group of Y are: Bzl, Cl_2 Bzl, Br-2, benzyl oxy carbonyl, acetyl, and Tos groups.

Examples of the protective group for thiol group of C are: HBzl, Bzl, and p-methyl benzyl groups.

Carboxyl group of E is protected by turning it into ester with benzyl alcohol, methanol, ethanol, and tert- butanol.

Examples of the activated carboxyl groups are: corresponding acid chloride, acid anhydride or mixed acid anhydride, azide, active ester (ester with pentachloro phenol, p- nitrophenol, N- hydroxy succine imide, N- hydroxy benzotriazole, and N- hydroxy- 5-norvolnen- 2, 3- dicarboxy imide).

In the said methods, the condensation reaction between the reactive amino group and the reactive carboxyl group (peptide bonding forming reaction) can be carried out under the existence of the solvent. As the solvent, various types which are used for the peptide bonding forming may be employed. Examples are: dimethyl formamide (DMF), dimethyl sulfoxide (DMSO), pyridine, chloroform, dioxane, dichloromethane, tetrahydrofuran (THF), ethyl acetate, N- methyl pyrrolidone, hexamethyl phosphoric triamide (HMPA), and their mixed solvents. The usage ratio for both the raw material compounds is not limited to any specifics. Usually, the ratio of one and the other is the same molar ratio to 5 times larger molar ratio, more desirably from the same molar ratio to 1.5 times larger molar ratio. The reaction temperature is appropriately selected from the range usually used for the peptide bonding forming reaction, generally from about -40 °C to about 60 °C, more desirably about -20 °C to about 40 °C. The reaction time is generally from several minutes to 30 hours.

The mixed acid anhydride method is carried out within the appropriate medium under the existence of basic compound, by using alkyl halo- carboxylic acid such as methyl chloro- formic acid, methyl bromo- formic acid, ethyl chloro- formic acid, ethyl bromo-formic acid, and iso- butyl chloro- formic acid. As the basic compounds, organic bases such as triethyl amine, trimethyl amine, pyridine, dimethyl aniline, N- methyl morpholine, 1, 5- diaza bicyclo (4, 3, 0) nonene- 5 (DBN), 1, 5- diaza bicyclo (5, 4, 0) undecene- 5 (DBU), and 1, 4- diaza bicyclo (2, 2, 2) octane (DABCO); and inorganic bases such as potassium carbonate, sodium carbonate, potassium hydrogen carbonate, and sodium hydrogen carbonate may be employed. As the solvent, various solvents commonly used for the mixed acid anhydride method may be used. Examples are: halogenized hydrocarbon types such as methylene chloride, chloroform, and dichloroethane; aromatic hydrocarbon types such as benzene, toluene, and xylene; ethers such as diethyl ether, THF, and dimethoxy ethane; esters such as methyl acetate and ethyl acetate; and non-protonic polar solvents such as DMF, DMSO, and HMPA. The reaction is usually carried out at -20 to 100 °C, more desirably at -20 to 50 °C, and the reaction time is generally from 5 minutes to 10 hours, more desirably from 5 minutes to 2 hours.

Further, the azide method is carried out by reacting the activated carboxyl group, for example, the carboxyl group activated by alcohols such as methyl alcohol, ethyl

alcohol, and benzyl alcohol, with hydrazine hydrate within an appropriate solvent. Examples of the applicable solvent are: dioxane, DMF, DMSO, and their mixed solvent. The usage amount of hydrazine hydrate is larger than the activated carboxyl group generally by 5 to 20 times in molar ratio, more desirably generally by 5 to 10 times in molar ratio. The reaction is usually carried out at most 50 °C, more desirably at -20 to 30 °C. By the said procedure, the compounds of which carboxyl groups are substituted by hydrazine (hydrazine derivatives) can be manufactured.

The compounds of which carboxyl groups are substituted by azide are manufactured by reacting the hydrazine derivatives obtained above with nitrite compounds under the existence of acid within the appropriate solvent. Hydrochloric acid is usually employed as the acid. Examples of the solvent are: dioxane, DMF, DMSO, and their mixed solvent. Further, sodium nitrite, iso-amyl nitrite, and nitrosyl chloride are used as the nitrite compound. Such the nitrite compound is employed at the amount of the same to double molar ratio, more desirably from the same to 1.5 times larger molar ratio, against the hydrazine derivative. The reaction is usually carried out at -20 to 0 °C, more desirably at -20 to -10 °C, and the reaction usually completes within 5 to 10 minutes.

Here, the peptide bonding forming reaction may be carried out under the existence of the condensation agent such as carbodiimide reagents (dicyclohexyl carbodiimide (DCC) and carbodiimidazole) and tetraethyl pyrophosphine.

When the elimination of the protective group is necessary at the each step of the said reaction process and at the final process, it is carried out by following the ordinary elimination reaction. Examples of such the methods are: the hydrogen addition by using catalysts such as palladium and palladium black; the reduction method by sodium metal within liquid ammonia; and the acidolysis by strong acids such as trifluoro acetic acid, hydrochloric acid, hydrofluoric acid, methane sulfonic acid, and hydrobromic acid. The said hydrogen addition by using catalyst can be carried out, for example, at 1 atmosphere of hydrogen pressure and at 0 to 40 °C. The usage amount of catalyst is usually 100 mg to 1 g and the reaction generally completes in 1 to 48 hours. Further, the said acidolysis is carried out usually at 0 to 30 °C, more desirably at 0 to 20 °C without any solvents by taking about 15 minutes to 1 hour. The usage amount of acid is usually 5 to 10 times of the raw material compounds. When only the protective group of amino acid is eliminated by the said acidolysis, the use of trifluoro acetic acid or hydrochloric acid as acid is desirable. Further, for the reduction by sodium metal within the liquid ammonia, sodium metal of which reaction solution presents the permanent blue for 30 seconds to 10 minutes should be employed and the reaction can be carried out at -40 °C to -70 °C.

The peptide of the present invention manufactured as above is isolated and purified from the reaction mixture by the peptide separation measures such as extraction, partition, and reverse phase high speed liquid chromatography.

The peptide of the present invention obtained as above is useful for the manufacturing of the antigen used for the manufacturing of the said anti-ED-B antibody.

Such the antigen is obtained by using the said peptide of the present invention as a hapten, which is bound to the ordinary carrier protein directly or through an appropriate spacer. This bonding reaction is executed by the general method employing the ordinary hapten- carrier bonding reagents.

In the above process, various compounds having at least two functional groups for the said bonding within one molecule may be utilized as a spacer. This also includes a part of the hapten- carrier bonding reagents. Examples of the said suitable spacers are: amino acids such as cysteine, lysine, and glycine, and particularly cysteine. These spacers can arbitrarily bind to N terminal and/or C terminal of the peptide of the present invention, therefore, the present invention also offers such the peptide further having the amino acids.

The reaction of the said amino acids as spacer and the hapten follows the said manufacturing of the peptide of the present invention.

Further in the said manufacturing method of the antigen, natural or synthetic polymer proteins which are commonly used for the preparation of the ordinary antigen are widely utilized as the carrier protein. Examples of the said carrier are: animal serum albumin such as horse serum albumin; bovine serum albumin, rabbit serum albumin, human serum albumin, and sheep serum albumin; animal serum globulin such as horse serum globulin, bovine serum globulin, rabbit serum globulin, human serum globulin, and sheep serum globulin; animal thyroglobulin such as horse thyroglobulin, bovine thyroglobulin, rabbit thyroglobulin, human thyroglobulin, and sheep thyroglobulin; animal hemoglobin such as horse hemoglobin, bovine hemoglobin, rabbit hemoglobin, human hemoglobin, and sheep hemoglobin; animal hemocyanin such as keyhole limpet hemocyanin (KLH); protein extracted from roundworm (ascaris extract, which is described in the following or which is further purified: JP Kokai 56-16414, J. Immun., 111, 260 - 268 (1973), J. Immun., 122, 302 - 308 (1979), J. Immun., 98, 893 - 900 (1967), and Am. J. Physiol., 199, 575 - 578 (1960)); poly-lysine, poly-glutamic acid, lysine- glutamic acid copolymer, and copolymer comprising lysine or ornithine.

As the hapten- carrier bonding reagent, those commonly used for the antigen preparation are widely utilized. The concrete examples are the following dehydration condensation agents: diazonium compounds such as bis- diazodized benzidine (BDB) and bis- diazodized- 3, 3- dianisidine (BDD) which bind tyrosine, histidine, and tryptophan through crosslinking; aliphatic group di- aldehydes such as glyoxal, malone di-aldehyde, glutal aldehyde, succine aldehyde, and adipo- aldehyde which bind amino group and amino group through crosslinking; di-maleimide compounds such as N, N'- o- phenylene dimaleimide and N, N'- m- phenylene dimaleimide which bind thiol group and thiol group through crosslinking; maleimide carboxyl- N- hydroxy succine imide esters such as methamaleimide benzoyl- N- hydroxy succine imide ester, 4- (maleimide methyl)- cyclohexane- 1- carboxyl- N'- hydroxy succine imide ester, N- succinimidyl -3-

(2- pyridyl dicyclo) propionate (SPDP) which bind thiol group and amino group through crosslinking; and carbodiimide type such as N, N-dicyclohexyl carbodiimide (DCC), N-ethyl- N'- dimethyl amino carbodiimide, 1- ethyl- 3- di-isopropyl amino carbodiimide, and 1- cyclohexyl- 3- (2- morpholinyl- 4- ethyl) carbodiimide, which are utilized for the ordinary peptide bonding formation reaction binding the amino group and carboxyl group by amide bonding. Further as the said hapten- carrier binding reagent, the combination of diazonium aryl carboxylic acids such as p- diazonium phenyl acetic acid with the ordinary peptide bonding forming reagent such as the said dehydration condensation agents may be utilized.

The manufacturing method of the antigen utilizing the said hapten, carrier protein, hapten- carrier bonding reagent, and spacer may follow the normal method. The reaction is generally carried out in aqueous solution or in the ordinary buffer solution with pH of 5 to 10, more desirably pH of 6 to 9, at 0 to 40 °C, more desirably at the vicinity of room temperature. The said reaction usually completes in about 2 to 5 hours.

In the said reaction, the usage ratio of the hapten, hapten- carrier bonding reagent, and the carrier may be appropriately determined. Usually, the carried is used at the amount of 0.5 to 5 times in weight, more desirably 1 to 2 times in weight, of the hapten, and the hapten- carrier bonding reagent is used at 1 to 30 times larger molar ratio. By the said procedure, the desired immunogen comprising the hapten- carrier composite in which carrier is bound to hapten directly or through spacer, is obtained.

The antigen obtained by the completion of the reaction is easily isolated and purified by following the normal methods such as the dialysis, gel filtration, and fractional precipitation methods.

Further, the ED-B peptide of the present invention can be utilized as the tracer used in the radio immunoassay (RIA) method or enzyme immunoassay (EIA) method by introducing radioactive substances such as ^{125}I and ^{131}I and various enzymatic reagents such as peroxidase (POX), chymotrypsinogen, procarboxy peptidase, glycero-aldehyde- 3- phosphate dehydrogenase, amylase, phosphorylase, D- Nase, P- Nase, β-galactosidase, glucose- 6- phosphate dehydrogenase, and ornithine decarboxylase. The introduction of the said radioactive substances can be carried out by the ordinary method. For example, radioactive iodine may be introduced by the oxidative iodination utilizing chloramine T (see N. H. Hunter and F. C. Greenwood; Nature, 194, 495 (1962), and Biochem. J., 89, 144 (1963)), and the introduction of enzymatic reagent may be carried out by the ordinarily known coupling methods such as the method by B. F. Erlanger (Acta. Endocrinol. Suppl. 168, 206 (1972)) and the method by M. H. Karol (Proc. Natl. Acad. Sci., USA., 57, 713 (1967)).

In the said methods, the peptide of the present invention which does not comprise tyrosine within its amino acid sequence may be arbitrarily bound with tyrosine at its N terminal and/or C terminal so that it is more advantageous at the said iodination.

And the present invention also offers the said ED-B peptide which is bound with such tyrosine.

[Examples]

Below, in order to interpret the present invention in further details, the manufacturing examples of the peptide of the present invention, manufacturing examples of the immunogen from the said peptide, and the manufacturing example of the antibody from the said antigen are presented. However, the present invention is not limited to these.

(Peptide Manufacturing)

Manufacturing Example 1

Manufacturing of the peptide of the present invention expressed by the formula (1)

The peptide synthesis was carried out by using Peptide Synthesizer Model 430A manufactured by Applied Biosystems.

By using Boc- cysteine- Pam resin (1% divinyl benzene cross-linked polystyrene, 0.559 mmol/g) as the starting raw material resin, Boc- amino acid derivatives listed below were introduced in this order from the C terminal through the peptide synthesis reaction by following the program constructed with the activator vessel, concentrator vessel, and the reaction vessel shown in Table 1 below.

Boc-L-E(OBzl)
Boc-L-G
Boc-L-I·1/2H_2O
Boc-L-P
Boc-L-F
Boc-L-D(OBzl)
Boc-L-V
Boc-L-S
Boc-L-Y(Br-Z)

Table 1

Activator vessel (1) Reference acid anhydride method or active ester method for Boc- amino acid derivatives Boc- amino acid derivatives 2 mmol DCC 1 mmol After stirring in CH_2Cl_2 for 2 to 8 minutes and removing the undissolved product, the content is transferred to the concentrator vessel.
Concentrator vessel (2) After filtration and condensation in nitrogen gas for 12 to 16 minutes, the solvent is substituted from CH_2Cl_2 to DMF.

Reaction vessel
(3) Elimination of Boc by 60% TFA/ CH_2Cl_2 (for 15 to 20 minutes)
(4) Washing with CH_2Cl_2 (x 3 times)
(5) Neutralization with di-isopropyl ethyl amine (x 2 times)
(6) Washing with DMF (x 6 to 8 times)
(7) Coupling reaction - Stirring for 60 minutes
(8) Washing with DMF (x 3 times) and CH_2Cl_2 (x 6 times)

By following the said program, the peptide chain was extended in sequence. After the final condensation reaction, the protective peptide resin was treated with HF at 0 °C for an hour under the existence of anisole and ethyl methyl sulfide and the protective group and resin were removed. Then, the product was washed with ethyl acetate and chloroform alternatively, dried, extracted with 2 M acetic acid, and freeze-dried to obtain the desired peptide.

Yield: Protective peptide resin - 1.7 g
Coarse peptide - about 400 mg

Then, the coarse peptide was purified by HPLC under the condition listed below.
Column: YMC pack D- ODS- 5 (2.1 x 25 cm)
Elution solution: 0.1% TFA/ 0.075% TFA, 99.9% CH_3CN
70/30 → 40/60 (an hour)
Detection: 206 nm
Flow rate: 5 ml/min

As a result, 250 mg of the purified peptide (below referred to as "Peptide B-1") was obtained (yield from coarse peptide: 63%).

Physical properties of the obtained Peptide B-1 are shown below.

Analysis by HPLC:
The result of HPLC using Synchropack RP 8 (4.1 x 250 mm) (Elution solution: 0.1% TFA/ 0.075% TFA - 99.9% CH_3CN = 100/0 → 40/60 (for 35 minutes, detection at 206 nm, flow rate of 2.0 ml/min) is shown in Figure 1.

In this figure, the ordinate indicates the absorbance (OD) at 206 nm (shown by solid line) and the gradient of CH_3CN (0 → 60%: shown by dotted line) and the abscissa indicates the elution time (minutes).

Amino acid analysis value:
Table 2 presents the amino acid analysis results obtained by using Hitachi L-8500 model.

Table 2

Amino Acids	Analytical Value (mol)	Expected Value
Asp	1.97 (2)	2
Ser	1.79 (2)	2
Glu	2.06 (2)	2
Gly	1.96 (2)	2
Cys	0.16 (1)	1
Val	2.01 (2)	2
Ile	1.95 (2)	2
Tyr	1.00 (1)	1
Phe	1.92 (2)	2
Pro	1.40 (1)	1

The said table displays the Tyr value as 1.00 mol. Further the value within the parenthesis at the analytical value column indicates the integer ratio.

Here, the said amino acid analysis value is the result measured after the hydrolysis with 6N- hydrochloric acid (110 °C, 24 hours).

Manufacturing Example 2

As similarly in Manufacturing Example 1, the peptide of the present invention expressed by the said formula (2) (below referred to as "Peptide B-2") was manufactured.

Its analytical pattern by HPLC is shown in Figure 2 as similarly to Figure 1.

Further, its amino acid analysis value is shown in Table 3 below (analyzed by Hitachi L- 8500 model).

Table 3

Amino Acids	Analytical Value (mol)	Expected Value
Asp	2.03 (2)	2
Thr	1.84 (2)	2
Ser	0.68 (1)	1
Glu	1.08 (1)	1
Gly	2.03 (2)	2
Cys	0.11 (1)	1
Val	0.93 (1)	1
Ile	2.00 (2)	2
Tyr	1.01 (1)	1
Phe	2.06 (2)	2
Pro	0.93 (1)	1

The said table displays the Ile value as 2 mol. Further the value within the parenthesis is the same as above.

(Manufacturing of antigen (immunogen))

Manufacturing Example 1

Peptide B-1 (5 mg) obtained in the peptide synthesis example 1 was dissolved in 1.0 mol of 0.1 M sodium phosphate buffer (pH 7.5). This solution was further added with 1 ml of the said sodium phosphate buffer comprising 10 mg of KLH. Then 1 ml of 20 mM glutal aldehyde solution was gradually added to this by dropwise and the reaction was carried out at room temperature by stirring for 30 minutes. The obtained reaction product was dialyzed at 4 °C for two days by using phosphate butter physiological salt solution (PBS) and the desired antigen (Antigen- 1) was obtained.

Manufacturing Example 2

By using Peptide B-2 obtained in the peptide synthesis example 2, the desired antigen (Antigen- 2) was obtained by the similar method as in the said Manufacturing Example 1.

(Manufacturing of the antibody of the present invention)

Manufacturing Example 1

Manufacturing of polyclonal antibody

The antigen obtained in the antigen manufacturing example (1 : 1 mixture of Antigen 1 and Antigen 2) (0.5 ml) was diluted with 1.0 ml PBS, then mixed and emulsified with 1.5 ml of adjuvant (complete Freund's adjuvant). This (1.0 ml) was endermically administrated to each of two rabbits by dividing into 30 to 40 sections on the back. Three more additional immunizations were similarly carried out at two week intervals. Then, the production of the desired antigen by both the test animals was confirmed by the immunoblot method.

After a week since the last immunization, the exsanguination was executed from the test animals. After leaving this at 4 °C for overnight, the centrifugal separation resulted in the desired antibody (anti-serum) of the present invention.

Manufacturing Example 2

Manufacturing of monoclonal antibody

The antigen obtained in the antigen manufacturing example (1 : 1 mixture of Antigen 1 and Antigen 2) (0.05 ml) was diluted with 0.5 ml PBS, then mixed and emulsified with 0.5 ml of adjuvant (complete Freund's adjuvant). This (0.2 ml) was hypodermically administrated to each of five BALB/c type mice (8 week old) by dividing into 5 to 10 sections on the back. Four more additional immunizations were similarly carried out at two week intervals. After 3 days since the last immunization, spleen cells were collected from spleen of each mouse and red blood cells existing in the said cells were liquefied and eliminated by the treatment with 0.83% ammonium chloride solution at 4 °C for 1 to 2 minutes. The cells obtained as above were used as the sensitized lymph cells and washed with the RPMI-1640 medium for several times.

On the other hand, HGPRT defect BALB/c originated P3U1 cell was sub-cultivated in the RPMI- 1640 medium comprising 15% FCS and further added with 100 μm of 8- azaguanine. This was used as myeloma cell.

The said myeloma cells (5×10^7 units) were mixed with 5×10^8 units of the sensitized cells prepared as above. The obtained cell mixture was centrifuged at 500 x g, then the cell fusion was carried out by adding 4 ml of 35% polyethylene glycol 1500 (Wako Junyaku) to this.

Hybridmer multiplied in the HAT medium was cloned by the limiting dilution method (Method in Enzymology, 73, 3 (1981)) by using BALB/c type mouse thymocyte as the feeder cell.

The search for the clone which produces the aimed antibody by the ELISA method utilized the reactivity with the cell type FN purified from the culture supernatant of the cell WI- 38 VA13 which is the human normal fibroblast WI-38 deteriorated by tumor virus SV 40, as the index. The said cloning was carried out four times while confirming no reactivity with the plasma type FN and the desired monoclonal antibody was obtained.

One of the desired monoclonal antibody producing hybridmers is called as "OAL-CF525". This was deposited to the Micro-organism Industrial Research Center, Industrial Technology Agency, Ministry of International Trade and Industry, with the code of "OAL- CF525" and its deposit number is "Micro-organism Industrial Research Center Bacterial Deposit No. 10052 (FERM P- 10052)".

The subclass of the antibody of the present invention "OAL- CF525" produced by the said hybridmer was determined by the Ouchterlony method.

As a result, the subclass of the said antibody was IgG_{2a}.

(Reactivity of the antibody of the present invention towards various types of FN)

(A) Reactivity of the antibody (anti-serum) of the present invention obtained in the said antigen manufacturing example 1 towards various types of FN was studied as below.

As the FN samples, the following four types were employed: (1) the plasma type FN (P) purified from human plasma, (2) the cell type FN (nc) purified from the culture supernatant of the human normal fibroblast WI-38, (3) the cell type FN (tc) purified from culture supernatant of the cell WI-38 VA 13 which is the said WI-38 deteriorated by tumor virus SV 40, and (4) the fetus organ type FN (f) purified from human placenta.

For these FNs, SDS-gel electrophoresis was carried out by using 6% polyacryl-amide gel. Further, the separated protein was transcribed on a nitrocellulose film by the electroblotting.

At first, these were photographed after dyeing the protein by using Fast Green.

In addition, the said nitrocellulose film was reacted with the antibody of the present invention which was diluted to 1000 times with the PBS comprising 2% bovine serum albumin (BSA) at room temperature for 2 hours, then washed with the PBS comprising 0.1% BSA for three times. Further this was reacted within the PBS comprising 2% BSA and having protein A (about 2×10^6 cpm) labeled with ^{125}I for 30 minutes, washed with the PBS comprising 0.1% BSA for three times, and dried in the air. This was then contacted with X-ray film and the autoradiogram was obtained.

Figure 3 presents the said results. The left panel in Figure 3 presents the result after the said Fast Green dyeing, while the right panel in Figure 3 presents the said autoradiogram result. Further each line in the figure indicates each of the said FN samples.

The left panel of the said figure reveals that essentially the same amount of each FN is transcribed on the nitrocellulose film. Further, the right panel of the said figure clarifies that the antibody of the present invention does not react with plasma type FN (p) and reacts only with the cell type FN (nc and tc) and the fetus organ type FN (f). Further, it is apparent that the antibody of the present invention more strongly binds with the FN (tc) produced by the cell deteriorated by virus than the FN (nc) produced by the normal fibroblast and the fetus organ type FN (f). This result supports that the utilization of the antibody of the present invention can recognize the cancerous FN from other FNs.

(B) Further, the reactivity of the antibody of the present invention (monoclonal antibody "OAL- CF525") obtained in the said antigen manufacturing example 2 towards various types of FN was measured as below.

In other words, the reactivity of the antibody of the present invention to the FN purified from human plasma and to the cell type FN purified from the culture supernatant of WI-38 VA 13 was studied by the ELISA method by using a 96-hole plastic plate solidifying each of these. Here, the antibody has utilized the culture supernatant of "OAL- CF525" diluted with the RPMI- 1640 medium comprising 15% FCS.

The said results are shown in Figure 4.

In the figure, the ordinate indicates the OD at 492 nm and the abscissa indicates the dilution number of the antibody, and (1) and (2) are the results for the cell type FN and the plasma type FN, respectively.

The said figure apparently indicates that the antibody of the present invention does not react with the plasma type FN but reacts with the cell type FN (tc) in the amount dependent manner.

Then, as similarly to the section (A), the electrophoresis was carried out for four types of FNs by using 4 to 15% gradient polyacrylamide gel, and the electroblotting

transcribed to the nitrocellulose film. Then, the reactivity of these with the antibody of the present invention OAL- CF525 was studied. Here, the antibody of the present invention was used by diluting the culture supernatant to 50 times with the PBS comprising 2% BSA. Other conditions are the same as in the section (A).

The results of the said test are the same as shown in Figure 3. This apparently indicates that the antibody of the present invention OAL- CF525 has the similar reactivity as the polyclonal antibody obtained from rabbit shown in the said section (A).

[Simple Interpretation of Figures]

Figure 1 is a graph showing the HPLC analytical result of the peptide EDB-1 obtained in the manufacturing example 1 for the peptide of the present invention. Figure 2 is a graph showing the HPLC analytical result of the peptide EDB-2 obtained in the manufacturing example 2 for the peptide of the present invention. Figure 3 shows the photographs, in place of diagrams, which present the reactivity of the antibody obtained in the manufacturing example 1 for the peptide of the present invention towards various FNs, studied by PAGE- SD3 gel electrophoresis and autoradiogram. And Figure 4 is the graph presenting the reactivity of the antibody obtained in the manufacturing example 2 towards various FNs.

Notes from the Translator

1. P. 14, line 12 (p. 5, bottom)
The word "ateitive" could not be located in any dictionaries and is translated on a best efforts basis.

2. P. 16, lines 13-14 (p. 6, middle)
The word "sulfenyl" may be typographical error of "sulfonyl", "sulfanyl" or "sulfinyl".

Figure 1

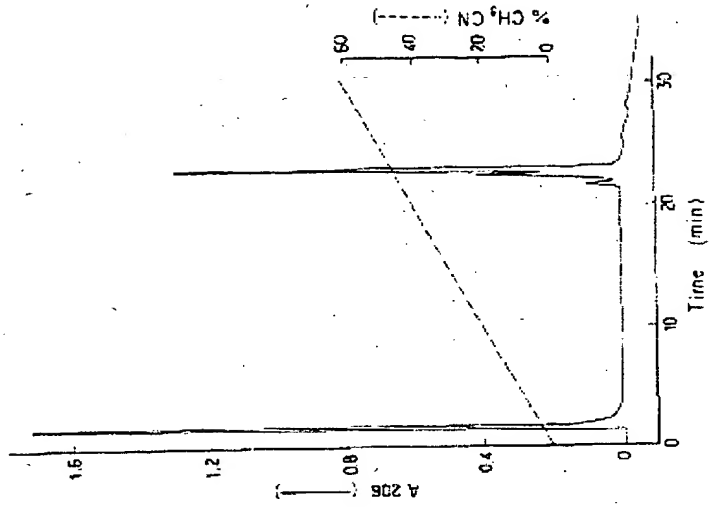

Figure 2

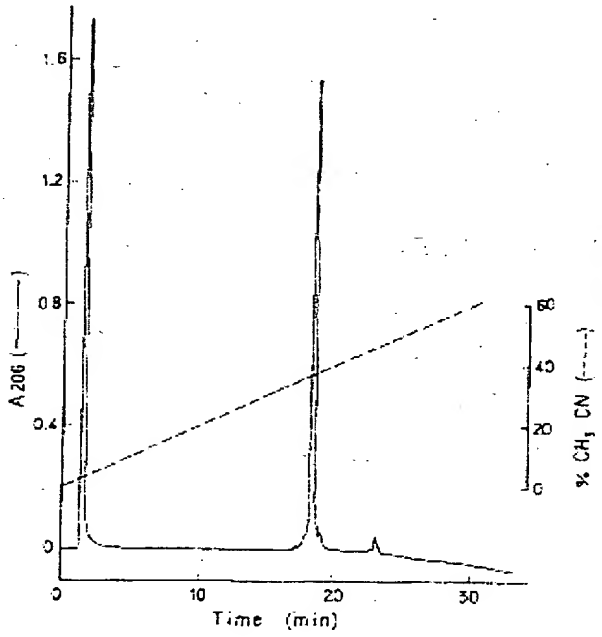

Figure 3

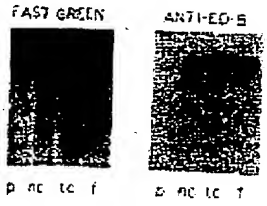

Figure 4